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<p>(21) International Application Number: PCT/EP96/02462</p> <p>(22) International Filing Date: 6 June 1996 (06.06.96)</p> <p>(30) Priority Data: 08/481,686 7 June 1995 (07.06.95) US</p> <p>(71) Applicants (for all designated States except AT DE): SANDOZ LTD. [CH/CH]; Lichtstrasse 35, CH-4002 Basel (CH). SYSTEMIX, INC. [US/US]; 3155 Porter Drive, Palo Alto, CA 94304 (US).</p> <p>(71) Applicant (for AT only): SANDOZ-ERFINDUNGEN VERWALTUNGSGESELLSCHAFT MBH [AT/AT]; Brunnerstrasse 59, A-1230 Wien (AT).</p> <p>(71) Applicant (for DE only): SANDOZ-PATENT-GMBH [DE/DE]; Humboldtstrasse 3, D-79539 Lörrach (DE).</p> <p>(72) Inventors: HILL, Beth, Louise; 780 Bond Way, Mountain View, CA 94040 (US). ROZLER, Elen; Apartment 130, 255 Rengstorff Avenue, Mountain View, CA 94040 (US). CHEN, Benjamin, P.; 2711 Parkside Drive, Fremont, CA 94536 (US).</p>	<p>(74) Common Representative: SANDOZ LTD.; Lichtstrasse 35, CH-4002 Basel (CH).</p> <p>(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p>Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>	
<p>(54) Title: METHODS FOR OBTAINING COMPOSITIONS ENRICHED FOR HEMATOPOIETIC STEM CELLS AND ANTIBODIES FOR USE THEREIN</p>		
<p>(57) Abstract</p> <p>A method for obtaining human hematopoietic stem cells is provided by enrichment for stem cells using a novel stem cell marker. Compositions obtained thereby and reagents for use therein are also provided.</p>		

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Methods for obtaining compositions enriched for hematopoietic stem cells and antibodies for use therein

The field of this invention is the isolation of a population of cells enriched for human hematopoietic stem cells.

Mammalian hematopoietic cells provide a diverse range of physiological activities. These cells are divided into lymphoid, myeloid and erythroid lineages. The lymphoid lineage, comprising B cells and T cells, provides for the production of antibodies, regulation of the cellular immune system, detection of foreign agents in the blood, detection of cells foreign to the host, and the like. The myeloid lineage, which includes monocytes, granulocytes, megakaryocytes as well as other cells, monitors for the presence of foreign bodies, provides protection against neoplastic cells, scavenges foreign materials, produces platelets, and the like. The erythroid lineage provides the red blood cells, which act as oxygen carriers.

Despite the diversity of the nature, morphology, characteristics and function of hematopoietic cells, it is presently believed that these cells are derived from a single precursor cell population, termed "stem cells." Stem cells are capable of self-regeneration and can become lineage committed progenitors which are dedicated to differentiation and expansion into a specific lineage. As used herein, "stem cells" refers to hematopoietic cells and not stem cells of other cell types.

A pluripotent stem cell can be defined as follows:
(1) gives rise to progeny in all defined hematolymphoid lineages; and (2) limiting numbers of cells are capable of fully reconstituting a seriously immunocompromised host in all

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blood cell types and their progenitors, including the pluripotent hematopoietic stem cell, by self-renewal.

A highly purified population of stem cells is necessary for a variety of *in vitro* experiments and *in vivo* indications. For instance, a purified population of stem cells will allow for identification of growth factors associated with their self-regeneration. In addition, there may be as yet undiscovered growth factors associated with: (1) the early steps of dedication of the stem cell to a particular lineage; (2) the prevention of such dedication; and (3) the negative control of stem cell proliferation.

Stem cells find use in: (1) regenerating the hematopoietic system of a host deficient in any class of hematopoietic cells; (2) a host that is diseased and can be treated by removal of bone marrow, isolation of stem cells and treatment with drugs or irradiation prior to re-engraftment of stem cells; (3) producing various hematopoietic cells; (4) detecting and evaluating growth factors relevant to stem cell self-regeneration; and (5) the development of hematopoietic cell lineages and assaying for factors associated with hematopoietic development.

Stem cells are important targets for gene therapy, where the inserted genes promote the health of the individual into whom the stem cells are transplanted. In addition, the ability to isolate stem cells can serve in the treatment of lymphomas and leukemias, as well as other neoplastic conditions where the stem cells are purified from tumor cells in the bone marrow or peripheral blood, and reinfused into a patient after myelosuppressive or myeloablative chemotherapy. Thus, there have been world-wide efforts toward isolating stem cells in substantially pure or pure form.

Stem cells constitute only a small percentage of the total number of hematopoietic cells. Hematopoietic cells are identifiable by the presence of a variety of cell surface

"markers." Such markers can be either specific to a particular lineage or progenitor cell or be present on more than one cell type. Currently, it is not known how many of the markers associated with differentiated cells are also present on stem cells. One marker, which was previously indicated as present solely on stem cells, CD34, is also found on a significant number of lineage committed progenitors. U.S. Pat. No. 4,714,680 describes a population of cells expressing the CD34 marker.

In view of the small proportion of the total number of cells in the bone marrow or peripheral blood which are stem cells, the uncertainty of the markers associated with the stem cell as distinct from more differentiated cells, and the general difficulty in assaying for stem cells biologically, the identification and purification of stem cells has been elusive. Characterizations and isolation of stem cells are reported in: Baum et al. (1992) Proc. Natl. Acad. Sci. USA 89:2804-2808; and Tsukamoto et al. U.S. Patent No. 5,061,620.

Decreased rhodamine 123 (rho123) staining of hematopoietic cells appears to correlate to stem cell potential. This so-called "rho¹⁰" marker is determined not by the initial dye accumulation but by an efflux process sensitive to P-glycoprotein (P-gp) inhibitors. Retention of several P-gp-transported fluorescent dyes, including rho123, in human bone marrow cells was inversely correlated with the expression of P-gp. Bone marrow cells expressing physical and antigenic characteristics of pluripotent stem cells show high levels of P-gp expression and fluorescent dye efflux. Fractions of human bone marrow cells isolated on the basis of either increased rho123 efflux or P-gp expression contain practically all the primitive progenitor cells of human bone marrow, including long-term culture-initiating cells (LTC-IC). Chaudhary and Roninson (1991) Cell 66:85-94.

Recently, the mouse stem cell has been obtained in at least highly concentrated, if not purified form, where fewer

than about 30 cells obtained from bone marrow were able to reconstitute all of the lineages of the hematopoietic system of a lethally irradiated mouse. Each assayed cell is multipotent for all hematopoietic lineages, while self-renewal is variable amongst these cells. Spangrude et al. (1988) Science 241:58-62; Smith et al. (1991) Proc. Natl. Acad. Sci. USA 88:2788-2792; Uchida (1992) Ph.D. Thesis Stanford U.; and see also, EPA 89 304651.6 and the references cited therein which describe the isolation of mouse stem cells.

Methods are provided to obtain compositions enriched for hematopoietic stem cells. The methods employ a separation regimen utilizing antibodies (α EM5) specific for a unique cell surface marker (EM5) that is expressed on stem and progenitor cells (EM5⁺), while being less accessible or absent on more mature cells.

Positive selection of stem cells with antibodies that recognize EM5 may be used in combination with selection for cells expressing other stem cell markers and/or negative selection with lineage-specific (LIN⁻) markers. Enriched populations of cells derived from these methods are also provided.

A hybridoma producing antibody to EM5 (SM27-588) was deposited with the American Type Culture Collection (ATCC), Rockville, Maryland / USA, on 2 June 1995, in accordance with the Budapest Treaty, and has been assigned accession number HB 11918.

It has now been found that the cell surface marker ("EM5") recognized by antibodies specific for EM5 (" α EM5") is expressed on hematopoietic stem cells. α EM5 subdivide the CD34⁺ cell population into approximately equal subpopulations, with the stem cell activity found predominantly in the EM5⁺ subpopulation. As described in detail in the examples section herein, α EM5 has the following characteristics. α EM5 binds 50% of CD34⁺ cells and 1-2% of total mononuclear cells on average.

α EM5 binds to a substantial subset of CD34⁺CD38⁻ cells. α EM5 binds a substantial subset of all CD34⁺Thy-1⁺ (both rho123^{lo} and rho132^{hi} subsets). α EM5 binds significant numbers of CD34⁺Thy-1⁻ cells. α EM5 does not bind significant numbers of CD34⁻ cells. Exemplary of an α EM5 antibody is the monoclonal antibody SM27-588 described in the Examples herein and produced by the deposited hybridoma.

Thus, α EM5 can be used in place of or in conjunction with antibodies to other stem cell markers, e.g., CD34, to purify the majority of progenitor cells and pluripotent stem cells from heterogeneous populations of hematopoietic cells. α EM5 binds to a larger subset of CD34⁺ cells than do mAbs to Thy-1. As described more fully in the examples section, EM5 defines a population of cells which is enriched in CFU-GM, in addition to the cobblestone area forming cell (CAFC) activity, which provides a desirable bone marrow graft composition. Further, based on SCID-hu thymus data, the functional properties of EM5⁺CD34⁺ cells can include T-cell progenitor activity.

The present invention thus encompasses methods of obtaining a composition substantially enriched in stem cells. The methods involve combining a mixture of hematopoietic cells with an antibody that recognizes and binds to EM5 under conditions which allow the antibody to bind to EM5 and separating the cells recognized by the antibody to obtain a population substantially enriched in EM5⁺ cells. The methods can be used as a diagnostic assay for the number of stem cells in a sample of hematopoietic cells. The cells and antibody are combined under conditions sufficient to allow specific binding of the antibody to EM5 and the EM5⁺ cells are then quantitated. The EM5⁺ cells can be isolated or further purified.

The results presented herein show that the EM5⁺ population includes the CD34⁺Thy-1⁺ population of cells which have previously been shown to be primitive pluripotential hematopoietic progenitors. Thus, EM5 is expressed on the subset of CD34⁺ cells which contains virtually all primitive

hematopoietic stem cells and α EM5 enables the purification of this subset of hematopoietic stem cells.

The methods can include further enrichment steps for cells by positive selection for other stem cell specific markers. Suitable positive stem cell markers include, but are not limited to, CD34⁺, Thy-1⁺, c-kit⁺. For examples, preferred cellular compositions include those consisting essentially of cells which are positive for ED5/CD34, ED5/Thy-1, or ED5/CD34/Thy-1. Preferably the EM5⁺ cells are human but can be derived from any suitable animal. By appropriate selection with particular factors and the development of bioassays which allow for self-regeneration of stem cells and screening of the stem cells as to their markers, a composition enriched for viable stem cells can be produced for a variety of purposes.

Preferably, the cells are subject to negative selection against one or more lineage specific markers, to remove cells whose expression of such markers evidences commitment to a specific lineage and retain those cells which are substantially negative in expression of such markers ("LIN⁻"). Methods of negative selection are known in the art. LIN⁻ cells generally refer to cells which lack significant expression of markers associated with T cells (such as CD2, 3, 4 and 8), B cells (such as CD10, 19 and 20), myeloid cells (such as CD14, 15, 16 and 33), natural killer ("NK") cells (such as CD2, 16 and 56), RBC (such as glycophorin A), megakaryocytes (CD41), mast cells, eosinophils or basophils. The absence or low expression of such lineage specific markers is identified by the lack of binding of antibodies specific to the cell specific markers, useful in so-called "negative selection". Preferably the lineage specific markers include, but are not limited to, at least one of CD2, CD14, CD15, CD16, CD19, CD20, CD38, HLA-DR and CD71; more preferably, at least CD14 and CD15. For example, a highly enriched composition of stem cells can be obtained by selective isolation to obtain a cellular composition consisting essentially of cells that are CD34⁺EM5⁺LIN⁻.

Table 1 summarizes probable phenotypes of stem cells in fetal, adult, and mobilized peripheral blood. In Table 1, myelomonocytic stands for myelomonocytic associated markers, NK stands for natural killer cells, FBM stands for fetal bone marrow, ABM stands for adult bone marrow, and AMPB stands for adult mobilized peripheral blood. As used herein, both infra, supra and in Table 1, the negative sign or superscript negative sign (⁻) means that the level of the specified marker is undetectable above Ig isotype controls by fluorescent activated cell sorting (FACS) analysis, and includes cells with very low expression of the specified marker.

Table 1

Probable Stem Cell Phenotypes

	NK and T cell markers				B cell markers				Myelomonocytic						Other						P-gp Activity
	CD2	CD3	CD8		CD10	CD19	CD20		CD14	CD15	CD16	CD33	CD34	CD38	HLA-DR	C-Kit	Thy	Rho			
FBM	-	-	-		-	-	-		-	-	-	?	+	-	+	+	+	lo	+		
ABM	-	-	-		-	-	-		-	-	-	-	+	-	lo/-	+	+	lo	+		
AMPB	-	-	-		-	-	-		-	-	-	lo/-?	+	?	lo/-	?	+	lo	+		

EM5⁺ stem cells can be isolated from any known source of stem cells, including, but not limited to, bone marrow, both adult and fetal, mobilized peripheral blood (MPB) and umbilical cord blood. The use of umbilical cord blood is discussed, for instance, in Issaragrishi et al. (1995) N. Engl. J. Med. 332:367-369. Initially, bone marrow cells can be obtained from a source of bone marrow, including but not limited to, ilium (e.g. from the hip bone via the iliac crest), tibia, femora, spine, or other bone cavities. Other sources of stem cells include, but are not limited to, embryonic yolk sac, fetal liver, and fetal spleen.

For isolation of bone marrow, an appropriate solution can be used to flush the bone, including, but not limited to, salt solution, conveniently supplemented with fetal calf serum (FCS) or other naturally occurring factors, in conjunction with an acceptable buffer at low concentration, generally from about 5-25 mM. Convenient buffers include, but are not limited to, HEPES, phosphate buffers and lactate buffers. Otherwise bone marrow can be aspirated from the bone in accordance with conventional techniques.

Various techniques can be employed to separate the cells by initially removing cells of dedicated lineage. Monoclonal antibodies are particularly useful for identifying markers associated with particular cell lineages and/or stages of differentiation. The antibodies can be attached to a solid support to allow for crude separation. The separation techniques employed should maximize the retention of viability of the fraction to be collected. Various techniques of different efficacy can be employed to obtain "relatively crude" separations. Such separations are where up to 10%, usually not more than about 5%, preferably not more than about 1%, of the total cells present not having the marker can remain with the cell population to be retained. The particular technique employed will depend upon efficiency of separation, associated cytotoxicity, ease and speed of performance, and necessity for sophisticated equipment and/or technical skill.

Procedures for separation can include, but are not limited to, magnetic separation, using antibody-coated magnetic beads, affinity chromatography, cytotoxic agents joined to a monoclonal antibody or used in conjunction with a monoclonal antibody, including, but not limited to, complement and cytotoxins, and "panning" with antibody attached to a solid matrix, e.g., plate, elutriation or any other convenient technique.

Separation techniques include, but are not limited to, those based on differences in physical (density gradient centrifugation and counter-flow centrifugal elutriation), cell surface (lectin and antibody affinity), and vital staining properties (mitochondria-binding dye rho123 and DNA-binding dye Hoechst 33342).

Techniques providing accurate separation include, but are not limited to, FACS, which can have varying degrees of sophistication, e.g., a plurality of color channels, low angle and obtuse light scattering detecting channels, impedance channels, etc.

In a first separation, typically starting with about $1 \times 10^{8-9}$, preferably at about $5 \times 10^{8-9}$ cells, α EM5 can be labeled with one fluorochrome, while the antibodies for the various dedicated lineages, can be conjugated to at least one different fluorochrome. While each of the lineages can be separated in a separate step, desirably the lineages are separated at the same time as one is positively selecting for EM5 and/or other stem cell markers. The cells can be selected against dead cells, by employing dyes associated with dead cells (including but not limited to, propidium iodide (PI)). Preferably, the cells are collected in a medium comprising 2% FCS.

The purified stem cells have low side scatter and low to medium forward scatter profiles by FACS analysis. Cytospin preparations show the enriched stem cells to have a size between mature lymphoid cells and mature granulocytes. Cells

can be selected based on light-scatter properties as well as their expression of various cell surface antigens.

While it is believed that the particular order of separation is not critical to this invention, the order indicated is preferred. Preferably, cells are initially separated by a coarse separation, followed by a fine separation, with positive selection with α EM5.

The foregoing separations result in cellular compositions consisting essentially of the cells desired. A "cellular composition consisting essentially of" a particular population of cells is understood to mean a population of cells in a suitable medium which is substantially homogeneous with respect to the indicated marker, e.g., at least 80% pure, usually at least 90% pure, preferably at least 95% pure. In particular, compositions wherein the cellular component is made up of at least 80%, usually at least 90%, or preferably at least 95% of EM5⁺ cells can be isolated in the manner described above, and are comprised within the scope of the invention. The desired stem cells can be further enriched by selection for LIN⁻ markers and/or Thy-1⁺ and/or rho¹⁰, or combinations of these markers as listed in Table 2, and being able to provide for cell regeneration and development of members of all of the various hematopoietic lineages. Note that the blank spaces in Table 2 do not mean that the cells are negative for the specified marker; they simply mean the marker is not used.

Table 2 Possible Combinations of Selections for Stem Cell Populations				
EM5 ⁺	CD34 ⁺	Thy ⁺	LIN ⁻	rho ^{lo}
+	+	+	+	+
+	+	+	+	
+	+	+		
+	+			
+	+			+
+		+		+
+		+	+	+
+			+	+
+			+	
	+	+	+	
	+	+	+	+
		+	+	+
	+	+		+
		+		+
		+	+	

By separating CD34⁺EM5⁺ cells from human hematopoietic sources, the long-term culture activity is enriched in the EM5⁺ fraction compared to EM5⁻. Moreover, the EM5⁺ cells will generate both B and myeloid cells in long-term cultures. In further enrichments of the EM5⁺ cells using antibodies to Thy-1 and/or any of the combinations specified in Table 2 and/or c-kit, the stem cell frequency can be further increased.

The cells obtained as described above can be used immediately or frozen at liquid nitrogen temperatures and stored for long periods of time, being thawed and capable of being reused. The cells will usually be stored in 10% DMSO, 50% fetal calf serum (FCS), 40% RPMI 1640 medium. Once thawed, the cells can be expanded by use of growth factors and/or stromal cells associated with stem cell proliferation and differentiation.

In another embodiment of the invention, a composition highly enriched in stem cells is provided. The results presented herein indicate that antibodies to EM5 recognize and bind with high specificity to a cell surface antigen found on human hematopoietic cells, and exposed to a high degree on stem cells. This specificity can be used to isolate and purify stem cells. Such a composition has utility in reconstituting hematopoietic systems and in studying various parameters of hematopoietic cells.

The compositions enriched for stem cells can be used in autologous hematopoietic engraftment, where the cells can be freed of neoplastic cells. Further, the use of autologous stem cells will avoid graft-versus-host disease. In addition, the cells can be modified by appropriate gene transfer to correct genetic defects or provide genetic capabilities naturally lacking in the stem cells or their progeny, either as to the individual or as to hematopoietic cells generally. In addition, the stem cell composition can be used to isolate and define factors associated with their regeneration and differentiation.

The cells generated from EM5⁺ cells and obtained from these cultures give rise to B cells, T cells, erythroid cells and myelomonocytic cells in the *in vivo* assays described below. In vitro analyses for hematopoietic progenitor cells have also been reported by Whitlock and Witte (1982) Proc. Natl. Acad. Sci. USA 79:3608-3612; and Whitlock et al. (1987) Cell 48:1009-1021.

In vivo demonstration of sustained hematopoietic ability of the isolated cell populations can be accomplished by the detection of continued myeloid, erythroid and B-lymphoid cell production in the SCID-hu bone model. Kyoizumi et al. (1992) Blood 79:1704; Chen et al. (1994) Blood 84:2497. To analyze this potential, one can isolate human fetal bone and transfer a longitudinally sliced portion of this bone into the mammary fat pad of a scid/scid animal: the bone cavity is depleted of endogenous progenitor cells by whole body irradiation of the mouse host prior to injection of the test donor population. The HLA of the population which is injected is mismatched with the HLA of the recipient bone cells.

To demonstrate differentiation to T cells, fetal thymus is isolated and cultured from 4-7 days at about 25°C, so as to deplete substantially the lymphoid population. The cells to be tested for T cell activity are then microinjected into the thymus tissue, where the HLA of the population which is injected is mismatched with the HLA of the thymus cells. The thymus tissue can then be transplanted into a scid/scid mouse as described in US Patent No. 5,147,784, particularly transplanting under the kidney capsule. Specifically, a sorted population of EM5⁺ cells can be microinjected into HLA mismatched thymus fragments. After 6-10 weeks, assays of the thymus fragments injected with EM5⁺ cells can be performed and assessed for donor-derived T cells.

The cell compositions can find use in a variety of ways. They can be used to fully reconstitute an immunocompromised host such as an irradiated host and/or a host subject to chemotherapy; or as a source of cells for specific lineages, by providing for their maturation, proliferation and differentiation into one or more selected lineages by employing a variety of factors, including, but not limited to, erythropoietin, colony stimulating factors, e.g., GM-CSF, G-CSF, or M-CSF, interleukins, e.g., IL-1, -2, -3, -4, -5, -6, -7, -8, etc., or the like, or stromal cells associated with the stem cells becoming committed to a particular lineage, or with

their proliferation, maturation and differentiation. The EM5⁺ cells can also be used in the isolation and evaluation of factors associated with the differentiation and maturation of hematopoietic cells. Thus, the invention encompasses the use of EM5⁺ cells in assays to determine the activity of media, such as conditioned media, or to evaluate fluids for cell growth activity, involvement with dedication of particular lineages, or the like.

The EM5⁺ cells can be used for the treatment of genetic diseases. Thus, the invention encompasses treatment of genetic diseases associated with hematopoietic cells by genetic modification of autologous or allogeneic stem cells to correct the genetic defect. For example, diseases including, but not limited to, β -thalassemia, sickle cell anemia, adenosine deaminase deficiency, recombinase deficiency, recombinase regulatory gene deficiency, etc. can be corrected by introduction of a wild-type gene into the EM5⁺ cells, either by homologous or random recombination. Other indications of gene therapy are introduction of drug resistance genes to enable normal stem cells to have an advantage and be subject to selective pressure during chemotherapy. Suitable drug resistance genes include, but are not limited to, the gene encoding the multidrug resistance (MDR) protein.

Diseases other than those associated with hematopoietic cells can also be treated by genetic modification, where the disease is related to the lack of a particular secreted product including, but not limited to, hormones, enzymes, interferons, growth factors, or the like. By employing an appropriate regulatory initiation region, inducible production of the deficient protein can be achieved, so that production of the protein will parallel natural production, even though production will be in a different cell type from the cell type that normally produces such protein. It is also possible to insert a ribozyme, antisense or other message to inhibit particular gene products or susceptibility to diseases, particularly hematolymphotropic diseases.

In another embodiment, the invention encompasses antibodies which specifically recognize the cell surface marker having the epitope recognized by SM27-588. As used herein, the term " α EM5" encompasses any antibody or fragment thereof, either native or recombinant, synthetic or naturally-derived, which retains sufficient specificity to bind specifically to the cell surface marker having the epitope recognized by SM27-588. As used herein, the terms "antibody" or "antibodies" include the entire antibody and antibody fragments containing functional portions thereof. The term "antibody" includes any monospecific or bispecific compound comprised of a sufficient portion of the light chain variable region and/or the heavy chain variable region to effect binding to the epitope to which the whole antibody has binding specificity. The fragments can include the variable region of at least one heavy or light chain immunoglobulin polypeptide, and include, but are not limited to, Fab fragments, F(ab')₂ fragments, and Fv fragments.

In addition, the monospecific domains can be attached by any method known in the art to another suitable molecule. The attachment can be, for instance, chemical or by genetic engineering. The α EM5 can be produced by any recombinant means known in the art. Such recombinant antibodies include, but are not limited to, fragments produced in bacteria and non-human antibodies in which the majority of the constant regions have been replaced by human antibody constant regions. In addition, such "humanized" antibodies can be obtained by host vertebrates genetically engineered to express the recombinant antibody.

As used herein, α EM5 includes monoclonal antibody SM27-588 or any monoclonal antibody or polyclonal antibody, that binds specifically to EM5 in such a manner as to recognize, preferentially, hematopoietic progenitor and stem cells. This also includes any α EM5 having the same antigenic specificity as SM27-588.

The α EM5 antibodies are obtained by methods known in the art for production of antibodies or functional portions

thereof. The actual methods used are described in the Examples presented herein although any method known in the art of antibody production can be used. Such methods include, but are not limited to, separating B cells with cell-surface antibodies of the desired specificity, cloning the DNA expressing the variable regions of the light and heavy chains and expressing the recombinant genes in a suitable host cell. Standard monoclonal antibody generation techniques can be used wherein the antibodies are obtained from immortalized antibody-producing hybridoma cells. These hybridomas can be produced by immunizing animals with stem cells, and fusing B lymphocytes from the immunized animals, preferably isolated from the immunized host spleen, with compatible immortalized cells, preferably a B cell myeloma.

The antibodies can be conjugated to other compounds including, but not limited to, enzymes, magnetic beads, colloidal magnetic beads, haptens, fluorochromes, metal compounds, radioactive compounds or drugs. The enzymes that can be conjugated to the antibodies include, but are not limited to, alkaline phosphatase, peroxidase, urease and β -galactosidase. The fluorochromes that can be conjugated to the antibodies include, but are not limited to, fluorescein isothiocyanate, tetramethylrhodamine isothiocyanate, phycoerythrin, allophycocyanins and Texas Red. For additional fluorochromes that can be conjugated to antibodies see Haugland, R. P. *Molecular Probes: Handbook of Fluorescent Probes and Research Chemicals* (1992-1994). The metal compounds that can be conjugated to the antibodies include, but are not limited to, ferritin, colloidal gold, and particularly, colloidal superparamagnetic beads. The haptens that can be conjugated to the antibodies include, but are not limited to, biotin, digoxigenin, oxazalone, and nitrophenol. The radioactive compounds that can be conjugated or incorporated into the antibodies are known to the art, and include but are not limited to technetium 99m (^{99}Tc), ^{125}I and amino acids comprising any radionuclides, including, but not limited to, ^{14}C , ^3H and ^{35}S . Kits, e.g., for assaying or isolating

hematopoietic stem cells, comprising α EM5, optionally labelled or conjugated as described above, are also comprised in the scope of the invention.

EXAMPLE 1

Cell Processing and Monoclonal Antibody ProductionCadaveric Bone Marrow and Mobilized Peripheral Blood

Cadaveric bone marrow cell suspensions derived from multi-organ donor vertebral bodies are obtained from Northwest Tissue Center (Seattle, WA). Patient peripheral blood samples are obtained after informed consent and chemotherapeutic regimens designed to mobilize primitive hematopoietic cells into the periphery. Multiple myeloma patients are mobilized with a high dose of cyclophosphamide and GM-CSF according to standard techniques. Non-Hodgkins lymphoma (NHL) patients are mobilized with VP16 and G-CSF.

Cells are separated over IsoPrep (Robbins Scientific, Sunnyvale, CA) harvesting the low density mononuclear cells ($\rho < 1.068$ g/mL for cadaveric bone marrow, $\rho < 1.077$ g/mL for peripheral blood) and are further stained with antibodies for FACS and analysis.

Production of Monoclonal Antibodies (MAbs)

C57/B1 mice are tolerized to CD34⁻ bone marrow cells by injection with cyclophosphamide (200 mg/kg) at 24 and 48 hours after interperitoneal injection with 10^7 CD34⁻ bone marrow cells. After 4 weeks, this tolerization regimen is repeated. Starting approximately 1 month later, mice are immunized interperitoneally with CD34⁺ cells (e.g. CD34⁺CD38⁻ at 0.5 to 1×10^6 cells/mouse) at 4 week intervals for a total of three immunizations. All immunizations are in RIBI adjuvant (RIBI Adjuvant Systems). Three days after the last immunization, the spleen is harvested to create hybridomas.

Splenocytes from immunized mice are fused to the myeloma partner P3xAg8.653 using standard PEG mediated fusion according to the method described by Galfre et al. (1977) Nature 266:550-552. Hybridomas from a single spleen are plated into 19 x 96 well tissue culture plates in RPMI + 10% FCS containing HAT to kill non-hybridoma cells. Fourteen days after fusion,

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supernatants from the hybridoma wells containing secreted antibodies are harvested for testing.

The hybridomas secreting SM27-588 are subcloned two to three times by limiting dilution in the presence of Hybridoma Enhancing Supplement (Sigma) to ensure monoclonality.

SM27-588, an antibody produced by this method, is shown to be IgM by an ELISA in which isotype-specific alkaline-phosphatase-labeled antibodies are used to detect SM27-588 captured in a microtiter well with immobilized anti-mouse Ig.

EXAMPLE 2

Antibody Staining, Fluorescence Activated Cell Sorting and Analysis

The buffer used in antibody staining is Dulbecco's modified phosphate buffered saline (Ca⁺⁺ and Mg⁺⁺ free) supplemented with 2% fetal bovine serum, 10 mM HEPES, 10 U/mL heparin, and 1 mg/mL human gamma globulin (Gamimune™, Miles, Elkhart, IN). Cells are incubated at 10⁷/mL in SM27-588 hybridoma supernatant diluted 1/2 in buffer for 30 minutes on ice. Cells are washed and SM27-588 binding is detected by adding PE-labelled rabbit anti-mouse IgM at 1/100 dilution (Zymed) and incubating for 30 minutes on ice. Cells are washed again and resuspended to 10⁷/mL in 2% normal mouse and 2% normal goat serum and incubated for 10 minutes on ice.

FITC or sulforhodamine-conjugated anti-CD34 antibody (Tük 3, F(ab')₂) is added at 3 µg/mL, and to amplify the PE signal, PE-labelled goat anti-rabbit IgG is added (Zymed, 1/100) and incubated 30 minutes on ice. Cells are washed and resuspended for cell sorting or analysis in buffer containing 1 µg/ml propidium iodide to stain non-viable cells. Cells are sorted and analyzed on a FACStar^{PLUS} (Becton Dickinson, San Jose, CA) equipped with two lasers, one emitting at 488 nm and a second (CR-599, Coherent, Palo Alto, CA) tuned to 600 nm to detect sulforhodamine fluorescence.

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Sort gates are established to collect cells that are sulforhodamine positive (CD34⁺), propidium iodide^{dim} (viable). These cells are further divided into subsets based upon the level of PE staining (EM5⁺ and EM5⁻). The sorted cells are used in the AC6.21 co-culture, methylcellulose and SCID-hu thymus assays described below.

Three color immunofluorescent labelling is performed on the FACSCAN, using Dulbecco's modified phosphate buffered saline as described above with the exception that 2% dialyzed BSA replaces the 2% FCS. Cells are stained with SM27-588 and detected with the double layer of PE-labelled antibodies as described above. Biotinylated anti-CD38 (Leu-17, Becton Dickinson) is added at the same time as the PE-goat anti-rabbit IgG. After incubation for 30 minutes and washing, FITC-labelled anti-CD34 and streptavidin Red613 (Gibco, 1/50 dilution) are added and incubated for 30 more minutes. Cells are washed a final time and the cell pellet is resuspended in buffer containing propidium iodide. 2000 FITC positive, low SSC events (CD34⁺ cells) are collected and displayed as FL2 SM27-588 or IgM isotype) versus FL3 (CD38⁺ for moderately bright, or dead for extremely bright). This FACS analysis shows that SM27-588 binds to a substantial subset of CD34⁺CD38⁻ cells.

For the five color analysis, cells are stained but not sorted. Cells are stained with rho123 (Molecular Probes, Eugene, OR) by incubating in buffer with 1 µg/mL rho123 for 30 minutes at 37°C, washing to remove excess rho123 and then incubating cells in buffer without rho123 for 30 minutes at 37°C to allow efflux. Subsequently, cells are stained at 4°C with SM27-588 or control IgM (detected with PE-labelled rabbit anti-mouse IgM, followed by PE-labelled goat anti-rabbit IgG, both from Zymed and diluted 1/100, FL2), anti-Thy-1 (GM201, detected with Texas Red-labelled goat anti-mouse IgG₁, FL5) and anti-CD34 (Tük 3, Cy5-labelled FL4) and propidium iodide (FL3). Rhodamine 123 staining is detected in the FL1 channel.

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The results obtained in the five color assay shows that SM27-588 binds to a substantial number of CD34⁺Thy-1⁺ cells (both rho123^{lo} and rho123^{hi} subsets). Further FACS analysis shows that α EM5 (SM27-588) subdivides CD34⁺ cells and binds nearly all CD34⁺Thy-1⁺ cells. The results indicate that α EM5 (SM27-588) binds an average of 50% CD34⁺ cells from bone marrow and 1-2% of total mononuclear cells on average. The results further indicate that α EM5 (SM27-588) does not bind significant numbers of CD34⁻ cells.

EXAMPLE 3

Staining of mobilized peripheral blood

Mobilized peripheral blood is obtained from a non-Hodgkins lymphoma (NHL) patient and a multiple myeloma patient and processed as described in Example 1. Cells are then stained with antibodies according to the method described in Example 2.

The results show that binding of SM27-588 to mobilized peripheral blood CD34⁺ cells is similar to that observed in bone marrow. SM27-588 binds 50-90% (avg. 69%, n=10) of CD34⁺ cells from mobilized peripheral blood. In 6 of 10 cases, the binding to CD34⁻ cells is less than 3%. In the remaining cases, binding to CD34⁻ cells is as high as 35%. It is uncertain whether binding to CD34⁻ cells is due to the variably "sticky" nature of these cells for fluorophor-labelled immunologic reagents. SM27-588 binds nearly all CD34⁺ Thy-1⁺ cells from mobilized peripheral blood.

EXAMPLE 4

Immunofluorescence Staining of Various Cell

Lines by α EM5

The expression of EM5 on a variety of cultured hematopoietic cell lines is tested by indirect immunofluorescence. All cultured cell lines are obtained from the ATCC. Human umbilical vascular endothelial cells (HUVEC) are obtained from Cell Systems, Kirkland, WA. Cell lines are

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immunolabelled with SM27-588 at 4°C for 30 minutes, washed and further incubated for 30 minutes and detected using PE-labelled rabbit anti-mouse IgM (Zymed). After washing twice, the cells are subjected to flow cytometric analysis using a FACSCAN. Analysis gates are set according to a non-binding isotype matched IgM control antibody.

For determination of neuraminidase sensitivity of the epitopes, HEL or bone marrow cells are incubated at 10^7 cells/mL with 0 to 500 mU/mL of neuraminidase for 1 hour at 37°C. In order to determine the glycoprotease sensitivity of the epitopes, cells are incubated at 10^7 cells/mL in HBSS and a 1/10 dilution of O-sialoglycoprotein endopeptidase (CLE100, Accurate Chemical). Cells are incubated at 37°C for 30 minutes. Cells are then washed and subsequently tested for antibody binding by indirect immunofluorescence. The results obtained are presented in Table 3 which shows the percent of EM5 positive cells for each cell line tested.

Peripheral blood mononuclear cells (PBL) and HUVEC are also tested for the expression of epitopes bound by α EM5. As a comparison, the HUVEC are first incubated with IL-1 β at 10 ng/ml for 4 hrs. IL-1 β activates some integrins on endothelial cells. The results obtained are presented in Table 3. The pattern of reactivity is not identified as similar to that described for mAbs to other antigens which have been described as expressed on CD34 $^+$ cells. Furthermore, no binding to PBL is observed. The level of epitope expression is modest on both bone marrow and other positively stained cells tested. The treatment of bone marrow cells with neuraminidase or glycoprotease does not diminish the binding of SM27-588 indicating that carbohydrate components are unlikely to be major aspects of the epitope defined by this mAb. The staining of HUVEC after activation with IL-1 β is no different from untreated cells.

Table 3

Cell Line	KG1	Jurkat	N417	HEL	HEL' neuraminidase	Daudi	Molt4	HL60	K562	U937	TFI	MCF7	T47D	HUVEC	HUVEC' IL-1 (4h)	PBL (ficolled)
n=3																
<5	<5	n=2	n=4	n=4	16	<5	<5	<5	n=2	<5	n=3	n=2	n=2	n=2	n=2	n=6
		<5	<5	8					50		<5	65	95	14	12	<3

EXAMPLE 5

Characterization of EM5⁺ CellsCo-culture

Sorted cell populations are analyzed by limiting dilution analysis for cobblestone area forming cell frequency by limiting dilution analysis according to the method described by Baum et al. (1989).

Briefly, CD34⁺ cadaveric bone marrow cells are sorted into CD34⁺EM5⁺ and CD34⁺EM5⁻ subsets as described in Example 1. A passage of AC6 (Whitlock et al. (1987) Cell 48:1009-1021), AC6.21, is used herein and is alternatively referred to as SyS1. Confluent AC6.21 stromal cell layers are maintained for 3-7 weeks without passage by changing of the tissue culture medium every 5-7 days. The culture is supplemented with human recombinant IL-6 (10 ng/mL) and LIF (20 ng/mL) to enhance the proliferation of adult bone marrow cells. The ability to give rise to both myeloid and B lymphoid progeny is determined after 6 weeks of coculture by staining with anti-CD19-FITC, and anti-CD15-FITC and anti-CD33-PE (all from Becton Dickinson) and analyzing on the FACSCAN. The results obtained from two separate sets of experiments are depicted in Table 4. The results show that the long term CAFC activity is enriched 30-90 fold in the double positive subset of CD34⁺ cells compared to the single positive subset (CD34⁺EM5⁻). Phenotypic analysis showed CD34⁺EM5⁺ cells are able to produce both lymphoid and myeloid cells in the SyS1 co-culture assay.

Methylcellulose Assay

Sorted cell populations are plated into methylcellulose cultures to determine colony forming cell activity according to the method described by Brandt et al. (1992) Blood 79:634. Growth factors are kit ligand (100 ng/mL), erythropoietin (2 U/mL), GM-CSF (10 ng/mL) and IL-3 ng/mL). The results in Table 4 show that α EM5 (SM27-588) binds the majority of day 14 CFU-GM and CFU-Mix but a minority of BFU-E.

SCID-hu thymus assay

Sorted cell populations are microinjected into depleted fetal thymic pieces and implanted under the kidney capsule of SCID-hu mice according to the method described by Galy et al. (1994) Blood 84:104-110. Six weeks after implantation, thymic pieces are recovered and analyzed for the presence of T cell progeny. The results obtained are depicted in Table 4.

Table 4
Functional Readouts of Cell Populations Subsetted With αSM27-588

Tissue	Sort Purity (Cross-contamination)	% Double Positive	Population Assayed	AC6.21 Results	CFC/10 ⁵ cells BFU-E/CFU-GM/CFU mix	SCID-hu thymus results
10362	2-5%	47%	CD34 ⁺ EM5 ⁺	1/7	400 / 1000 / 150	1/5
			CD34 ⁺ EM5 ⁻	1/610	3150 / 225 / 75	0/5
			CD34 ⁺	1/96	2150 / 1300 / 275	1/5
9041	5-7%	67%	CD34 ⁺ EM5 ⁺	1/28	3275 / 1975 / 150	
			CD34 ⁺ EM5 ⁻	1/825	177000 / 400 / 100	
			CD34 ⁺	1/35	6500 / 1225 / 50	

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be apparent to those skilled in the art that certain changes and modifications can be practiced. Therefore, the description and examples should not be construed as limiting the scope of the invention, which is delineated by the appended claims.

WHAT IS CLAIMED IS:

1. A cellular composition consisting essentially of cells which are EM5 positive.
2. The cellular composition according to claim 1 wherein the cells express at least one additional marker associated with stem cells.
3. The cellular composition according to claim 2 wherein the additional marker or markers are selected from the group consisting of CD34, Thy-1, P-gp and c-kit.
4. The cellular composition according to claim 1, 2, or 3 wherein the cells are negative for at least one lineage specific (LIN⁻) marker.
5. The cellular composition according to claim 4 wherein the cells are negative for at least one lineage specific (LIN⁻) marker selected from CD14, CD15, CD38, HLA-DR, CD71 and CD33.
6. A cellular composition according to claim 4 or 5 wherein the cells are negative for at least one lineage specific (LIN⁻) marker selected from CD2, CD16, CD19, CD20, or glycophorin A.
7. A cellular composition consisting essentially of hematopoietic cells and enriched for stem cells wherein the cells express EM5 and are negative for at least one lineage specific marker.
8. A cellular composition according to claim 7 wherein the cells are negative for at least one lineage specific (LIN⁻) marker selected from CD14 and CD15.
9. The cellular composition according to claim 7 or 8 wherein the cells are negative for at least one lineage

specific marker selected from CD2, CD16, CD19, CD20 and glycophorin A.

10. The cellular composition according to claim 7, 8, or 9 wherein the cells express at least one additional marker associated with stem cells.

11. The cellular composition according to claim 10 wherein the additional marker or markers is selected from the group consisting of CD34, Thy-1, c-kit and P-gp.

12. A cellular composition according to any one of claims 1 through 11 inclusive comprising at least one cell which has been transduced with a non-autologous gene.

13. A cellular composition according to any of claims 1 through 12 inclusive for therapeutic use.

14. Use of a cellular composition according to any of claims 1 through 12 inclusive in the manufacture of a composition for gene therapy.

15. A method of obtaining a composition substantially enriched in hematopoietic stem cells comprising the steps of:
combining a mixture of hematopoietic cells with an antibody that recognizes and binds to EM5 under conditions which allow the antibody to specifically bind to EM5; and
isolating cells recognized by the antibody to obtain a population of cells substantially enriched in EM5⁺ cells.

16. The method according to claim 15, further comprising the step of selecting the cells for expression of at least one additional marker associated with stem cells.

17. The method according to claim 16 wherein the additional marker is selected from the group consisting of CD34, Thy-1, P-gp and c-kit.

18. The method according to any one of claims 15 through 17 inclusive further comprising the step of selecting the cells for lack of expression of at least one lineage specific (LIN⁻) marker.

19. The method according to claim 18 wherein the cells are selected for lack of expression at least one lineage specific marker selected from the group consisting of CD14, CD15, CD38, HLA-DR, CD71 and CD33.

20. The method according to claim 18 or 19 wherein the cells are selected for lack of expression of at least one lineage specific marker selected from the group consisting of CD2, CD16, CD19, CD20 and glycophorin A.

21. A method of determining the stem cell content in a sample of hematopoietic cells comprising the steps of:

- a) combining a mixture of hematopoietic cells with an antibody that recognizes and binds to EM5 under conditions which allow the antibody to specifically bind to EM5; and
- b) quantitating the cells recognized by the antibody.

22. The method according to claim 21, further comprising the step of selecting the cells for expression of at least one additional marker associated with stem cells.

23. The method according to claim 19 wherein the additional marker is selected from the group consisting of CD34, Thy-1, P-gp and c-kit.

21. The method according to claim 18 further comprising the step of selecting the cells for lack of expression of at least one lineage specific marker.

22. The method according to claim 21 wherein the lineage specific marker is selected from the group consisting of CD14, CD15, CD38, HLA-DR, CD71 and CD33.

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23. The method according to claim 21 wherein the lineage specific marker is selected from the group consisting of CD2, CD16, CD19, CD20 and glycophorin A.

24. Antibody recognizing EM5.

25. Antibody according to claim 24 which binds specifically to the epitope recognized by the antibody SM27-588 produced by the hybridoma deposited with the ATCC under accession number HB 11918.

26. Antibody according to claim 25 which is SM27-588 produced by HB 11918 or its progeny.

27. A kit for isolating or assaying hematopoietic stem cells comprising an antibody according to any of claims 24, 25, or 26.

28. Use of an antibody according to any of claims 24, 25, or 26 in a method of isolating or assaying hematopoietic stem cells.

29. A hybridoma cell line capable of producing monoclonal antibody recognizing EM5.

30. A hybridoma cell line according to claim 29 which is HB 11918.

INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 96/02462

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N5/08 A61K35/28 A61K35/14 A61K48/00 G01N33/577
 C07K16/28 C12P21/08 C12Q1/24 C12N5/20 //(C12P21/08,
 C12R1:91)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N A61K G01N C07K C12P C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,95 03693 (SYSTEMIX, INC.) 9 February 1995 see page 7, line 1 - page 8, line 9; claims; table 1	1-30
A	WO,A,95 05843 (SYSTEMIX, INC.) 2 March 1995 see page 10, line 5 - line 16; claims	1-30

 Further documents are listed in the continuation of box C. Patent family members are listed in annex.

* Special categories of cited documents :

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INTERNATIONAL SEARCH REPORT

(information on patent family members)

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9503693	09-02-95	AU-A- 7476694	28-02-95
		CA-A- 2168348	09-02-95
		EP-A- 0711111	15-05-96

WO-A-9505843	02-03-95	AU-A- 7676994	21-03-95
		EP-A- 0722331	24-07-96
